Phospholipid association with the bovine cardiac mitochondrial adenosine triphosphatase

Rhoderick E. BROWN,* Rebecca I. MONTGOMERY, Priscilla I. SPACH and Carol C. CUNNINGHAM†

Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27103, U.S.A.

(Received 25 June 1984/Accepted 8 October 1984)

The association of different phospholipids with a lipid-depleted oligomycin-sensitive ATPase from bovine cardiac mitochondria [Serrano, Kanner & Racker (1976) *J. Biol. Chem.* **251**, 2453–2461] has been examined using three approaches. First, reconstitution of the ATPase with different synthetic diacyl phospholipids resulted in a 2–10-fold stimulation of ATPase specific activity depending upon the particular phospholipid employed. The phospholipid headgroup region displayed the following order of ATPase reactivation potential: dioleoylphosphatidylglycerol > dioleoylphosphatidic acid > dioleoylphosphatidylcholine. Furthermore, the ATPase showed higher levels of specific activity when reconstituted with dioleoyl phospholipid derivatives compared with dimyristoyl derivatives. Second, examination of the phospholipid remaining associated with the lipid-depleted ATPase upon purification showed that phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol were present. No relative enrichment of any of these phospholipids (compared with their distribution in submitochondrial particles) was noted. Therefore, no preferential association between the ATPase and any one phospholipid could be found in the mitochondrial ATPase. Third, the sodium cholate-mediated phospholipid exchange procedure was employed for studying the phospholipid requirements of the ATPase. Replacement of about 50% of the mitochondrial phospholipid remaining with the lipid-depleted ATPase could be achieved utilizing either synthetic phosphatidic acid or phosphatidylcholine. Examination of the displaced mitochondrial phospholipid showed that phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol were replaced with equal facility.

Previous studies on highly-purified preparations of the oligomycin-sensitive ATPase from beef heart mitochondria have clearly demonstrated

Abbreviations used: ATPase, adenosine triphosphatase; DCCD, dicyclohexylcarbodi-imide; PtdCho, egg phosphatidylcholine; PtdEtn, liver phosphatidylethanolamine; Ptd₂Gro, heart diphosphatidylglycerol; C_{18:1}PtdA, dioleoylphosphatidic acid; C_{18:1}PtdGro, dioleoylphosphatidylglycerol; C_{18:1}PtdCho, dioleoylphosphatidylcholine; C_{14:0}PtdGro, dimyristoylphosphatidylglycerol; C_{14:0}PtdCho, dimyristoylphosphatidylcholine.

* Present address: Department of Biochemistry, University of Virginia Medical School, Charlottesville, VA 22908, U.S.A.

† To whom reprint requests should be addressed.

the complex nature of this multi-subunit enzyme (Serrano *et al.*, 1976; Berden & Voorn-Brouwer, 1978; Galante *et al.*, 1979; Godinot *et al.*, 1981). All of these ATPase preparations can be isolated in forms exhibiting low activity even though residual mitochondrial phospholipid is known to be present. Several-fold increases in activity can be obtained by recombination of the lipid-depleted enzyme with additional phospholipid.

Earlier phospholipid reconstitution studies with the oligomycin-sensitive mitochondrial ATPase, performed with enzyme preparations of relatively low purity (Kagawa & Racker, 1966), have indicated that acidic phospholipids reactivate the lipid-depleted ATPase to higher levels of specific activity than do either neutral or basic phospho-

lipids (Bulos & Racker, 1968; Dabbeni-Sala *et al.*, 1974; Cunningham & George, 1975; Cunningham & Sinthusek, 1979). More recent studies (Brown & Cunningham, 1982a) using a highly-purified ATPase preparation (Serrano *et al.*, 1976) have supported these earlier findings. In the present investigation, the association of phospholipid with the ATPase preparation of Serrano *et al.* (1976) has been examined in further detail. The interaction of various synthetic acidic and neutral diacyl phospholipids with the ATPase has been studied in order to see how the phospholipid headgroup and hydrocarbon acyl chain regions influence both the specific activity and oligomycin sensitivity of the enzyme complex.

While the observations in the present and recent (Brown & Cunningham, 1982a) studies demonstrate the potential of acidic phospholipids to elicit increased activities from the lipid-depleted oligomycin-sensitive ATPase, they do not designate the immediate phospholipid environment of the multi-subunit complex. Therefore, in the present investigation, the reconstitution studies were augmented with an examination of the residual phospholipid associated with the highly-purified ATPase. These measurements, plus experiments in which the residual phospholipid was partially displaced with the cholate-mediated phospholipid exchange technique, have yielded information about the association of the ATPase with the different mitochondrial phospholipid classes. Preliminary results on portions of this work have been reported previously (Brown & Cunningham, 1979, 1982b).

Experimental procedures

Materials

Special enzyme grade $(\text{NH}_4)_2\text{SO}_4$ and sucrose were purchased from Schwarz/Mann, Orangeburg, NY, U.S.A.; ATP, phosphoenolpyruvate, oligomycin, DCCD, lyssolecithin, and phospholipase D (type I) from Sigma Chemical Co., St. Louis, MO, U.S.A.; crystalline bovine serum albumin from Miles Laboratories, IN, U.S.A.; pyruvate kinase and NADH from Boehringer Mannheim, Indianapolis, IN, U.S.A.; fatty acid methyl ester standards from Supelco, Inc., Bellefonte, PA, U.S.A.; $\text{C}_{18:1}\text{PtdA}$, $\text{C}_{18:1}\text{PtdGro}$, PtdCho , PtdEtn and Ptd_2Gro from Serdary Research Laboratories, London, Ontario, Canada; $\text{C}_{18:1}\text{PtdCho}$ from Calbiochem-Behring, La Jolla, CA, U.S.A.; asolecithin from Associated Concentrates, Woodside, NY, U.S.A.; and reagents for polyacrylamide-gel electrophoresis from Bio-Rad, Richmond, CA, U.S.A. The deoxycholic acid and cholic acid, obtained from Matheson, Coleman, and Bell, Norwood, OH, U.S.A., were recrystallized once in 70% ethanol before use.

$\text{C}_{14:0}\text{PtdGro}$ was synthesized from $\text{C}_{14:0}\text{PtdCho}$ (Sigma) and purified according to the procedure of Papahadjopoulos *et al.* (1973) with the following modifications. Washing of reactant products with NaCl and EDTA to remove calcium was repeated three times. Final purification of reaction products on the silicic acid column was carried out by using a stepwise elution of the column with 200 ml each of 5, 10, 15, 20, and 25% methanol in chloroform, followed by 600 ml of 30% methanol in chloroform, and finally with 400 ml of 35% methanol in chloroform. The $\text{C}_{14:0}\text{PtdGro}$ was eluted between 20 and 30% methanol in chloroform and appeared pure by t.l.c. The yield of $\text{C}_{14:0}\text{PtdGro}$ was 48%.

Analytical procedures

The protein concentrations of mitochondria and submitochondrial particles were determined by the biuret method (Gornall *et al.*, 1949) in the presence of 0.3% sodium deoxycholate. All other protein concentrations were determined by the method of Lowry *et al.* (1951) in the presence of 0.17% sodium deoxycholate. Crystalline bovine serum albumin was used as the standard. Lipid phosphorus determinations were carried out by the method of Chalvardjian & Rudnicki (1970). Sodium cholate was quantified by the method of Mosbach *et al.* (1954). Since low concentrations of sucrose (25–50 mM) interfered with this assay, the analyses were performed on samples of cholate extracted from the ATPase preparations by the procedure of Bligh & Dyer (1959).

The procedure of Laemmli (1970), slightly modified, was utilized for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The conditions were identical to Laemmli's except that 2 mM-EDTA was included in the gel and buffers, and the procedure was performed utilizing 1.5 mm slab gels according to the method of Douglas *et al.* (1979). The gel slab was a 10–15% linear gradient.

Isolation, assay, and phospholipid analysis of the oligomycin-sensitive mitochondrial ATPase

The beef heart submitochondrial particles utilized for preparation of the oligomycin-sensitive mitochondrial ATPase were made as described earlier (Cunningham & George, 1975). The mitochondrial ATPase was prepared as described by Serrano *et al.* (1976) with the following modifications. The precipitate (38–45 p ATPase) collected at 45% $(\text{NH}_4)_2\text{SO}_4$ saturation was rinsed twice with ATPase buffer (10 mM-Tris/ H_2SO_4 , pH 7.5, 0.5 mM-EDTA, 1.0 mM- MgSO_4 , 0.5 mM-dithiothreitol) before resuspension at 3 ml/g of original submitochondrial particles. This rinsing procedure removed residual cholate and phospholipid, and thus enhanced the dependence of the ATPase

activity on added phospholipid. The ATPase was further purified by centrifuging the 38–45p ATPase preparation on a linear sucrose gradient containing deoxycholate, lysolecithin, and $(\text{NH}_4)_2\text{SO}_4$ as described by Serrano *et al.* (1976). These steps removed a 32000Da polypeptide reported to be the adenine nucleotide transport protein (Serrano *et al.*, 1976), also yielding a preparation even further depleted of endogenous phospholipid. This fraction will be termed the gradient purified ATPase. Mitochondrial ATPase activity was measured as described previously (Brown & Cunningham, 1982a). In assay mixtures containing oligomycin, the inhibitor (5nmol) was added immediately after the phospholipid, but before initiation of the assay.

The phospholipids associated with the purified or reconstituted ATPase (0.5–2.0mg of protein) were extracted from the enzyme complex by the procedure of Bligh & Dyer (1959). The extracted phospholipids were separated by using two-dimensional t.l.c. (0.25mm silica gel H plates) as described by Cunningham *et al.* (1981) except that the 40°C heating step between chromatography in the first and second dimensions was omitted. The solvents used were chloroform/methanol/15.1M- NH_4OH (13:5:1, by vol.) for the first dimension and chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.) for the second dimension. Recovery of the phospholipids from the thin-layer plates was as described previously (Cunningham *et al.*, 1982). Quantification of the isolated phospholipids was performed by measuring their fatty acid concentrations by g.l.c. as outlined by Cunningham *et al.* (1982).

To determine the fatty acid composition of inner membrane PtdCho, PtdEtn, and Ptd₂Gro, submitochondrial particles prepared from inner membrane were extracted for their phospholipids which were subsequently analysed by the above procedure. The submitochondrial particles were prepared from digitonin-treated mitochondria (Kun *et al.*, 1979) as earlier described (Cunningham & George, 1975).

Reconstitution of the isolated ATPase with phospholipids

Purified phospholipids were dispersed in 10mM-Tris/ H_2SO_4 , pH 8.0, (0.2mg/ml) by bath sonicating with a Heat Systems ultrasonic sonifier (model 13) at an output of 240 W (25°C) for 5–10min in an N_2 atmosphere. Various amounts of the phospholipid dispersions were incubated with the isolated ATPase for 5min and the specific activity was measured both in the presence and absence of oligomycin (5nmol). Reconstitution of the ATPase with asolectin liposomes was performed similarly, with the liposomes (75mg/ml) being prepared by a

modification of the procedure of Conover *et al.* (1963). Sonication was performed for 30min at 0–4°C using a Heat Systems (model W185) sonifier (power output 70 W). The asolectin liposomes were stored under N_2 at 4°C and subsequently diluted in a solution containing 10mM-Tris/ H_2SO_4 , pH 7.7, 0.5mM-EDTA, 10mM-monothioglycerol, and 250mM-sucrose before being used in reconstitution procedures.

Detergent-mediated exchange of ATPase phospholipid for synthetic phospholipid

The detergent-mediated phospholipid exchange procedure (Warren *et al.*, 1974) was performed with the following modifications. Synthetic phospholipid (4mg) or asolectin (3.9mg) was suspended by bath sonication (see above) in a solution containing 10mM-Tris/ H_2SO_4 , pH 7.5, 1.0mM- MgSO_4 , 0.5mM-EDTA, 0.5mM-dithiothreitol, 100mM-sucrose, and a designated amount of sodium cholate. After addition of the ATPase (2mg), the mixture (0.3ml final volume) was incubated for 90min at 30°C under N_2 . Separation of unbound phospholipid and detergent from the ATPase was accomplished by centrifugation (300000g) for 90min at 30°C into discontinuous sucrose gradients. The sucrose gradients contained 2 and 3ml of 25 and 60% sucrose, respectively, plus ATPase buffer, but no sodium cholate.

The ATPase-phospholipid complex was recovered from the interface region of the gradient and gently dispersed with a 2ml glass/Teflon homogenizer. The ATPase was analysed for protein content and assayed for specific activity. The phospholipids bound to the ATPase were then extracted and quantified by g.l.c. (see above). The amount of phospholipid substitution in the ATPase using $\text{C}_{18:1}\text{PtdA}$ and $\text{C}_{18:1}\text{PtdCho}$ was calculated in the following manner. The amounts (nmol of fatty acid/mg of protein) of all the fatty acyl chains of the mitochondrial phospholipids which co-purified with untreated ATPase were quantified by comparing their peak areas with that of a known amount of internal standard, $\text{C}_{22:0}$. Then the same fatty acyl chain quantification procedure was applied to the synthetic phospholipid-substituted ATPase. The relative amount of phospholipid exchange was computed by comparing the absolute amounts (nmol of fatty acid/mg of protein) of the fatty acyl chains contributed exclusively by mitochondrial phospholipid in the untreated control ATPase versus that remaining in the phospholipid exchanged ATPase samples. In this manner, the simple addition of large amounts of extra phospholipid not normally found associated with ATPase could be distinguished from the actual replacement of mitochondrial phospholipid by synthetic phospholipid.

Results

Influence of phospholipid headgroup and acyl chain regions on ATPase specific activity and oligomycin sensitivity

The 38–45 p ATPase was reconstituted with various synthetic diacyl phospholipids at different protein-to-phospholipid ratios. The use of synthetic phospholipids allowed separate evaluation of the influence of the phospholipid polar headgroup and nonpolar acyl chain regions on ATPase specific activity and oligomycin sensitivity. Identical ATPase reconstitution experiments with asolectin provided a reference with which to compare the present work with earlier studies. As shown in Fig. 1, specific activities as high as 12.3 μmol of ATP hydrolysed/min per mg of protein, with 95% oligomycin sensitivity, were achieved with asolectin as an activator.

Initially, the effect of the phospholipid headgroup region on ATPase specific activity and oligomycin sensitivity was measured. Reconstitution of the ATPase with three different dioleoyl phospholipids produced dramatic alterations in levels of ATPase specific activity and indicated the following order of effectiveness: $C_{18:1}\text{PtdGro} > C_{18:1}\text{PtdA} > C_{18:1}\text{PtdCho}$ (Fig. 1a). Although measurements on the ATPase reconstituted with the same three dioleoyl phospholipids also showed dramatic alterations in

the oligomycin sensitivity, a different order of effectiveness was noted: $C_{18:1}\text{PtdA} > C_{18:1}\text{PtdCho} > C_{18:1}\text{PtdGro}$ (Fig. 1b). No direct correlation between headgroup charge and ATPase oligomycin sensitivity was evident. However, the alterations in oligomycin sensitivity must be attributed to the headgroup region since the phospholipid acyl chains were identical.

The phospholipid acyl chain region also affected ATPase specific activity and oligomycin sensitivity (Fig. 1). The dioleoyl derivative of phosphatidylglycerol elicited higher levels of ATPase specific activity than the dimyristoyl derivatives even though the assay temperature (30°C) was above the phase transition temperature of both lipids (Findlay & Barton, 1978). Similar results were obtained when the dioleoyl and dimyristoyl derivatives of phosphatidylcholine were compared (Brown & Cunningham, 1982b). The oligomycin sensitivity of the ATPase displayed complex behaviour following reconstitution with the dioleoyl and dimyristoyl derivatives of phosphatidylglycerol and phosphatidylcholine. The ATPase activity elicited by the dioleoyl derivative of phosphatidylglycerol was strikingly less sensitive to oligomycin than the dimyristoyl derivative (Fig. 1b). In contrast, no such differences in oligomycin sensitivity were observed with dioleoyl and dimyristoyl derivatives of phosphatidylcholine (Brown & Cunningham, 1982b).

Composition of the residual phospholipid associated with the ATPase upon purification

The fact that higher levels of ATPase specific activity were obtained following reconstitution with acidic phospholipids (Fig. 1) suggested that the enzyme, if functioning at maximal activity in the membrane, might reside in a negatively-charged phospholipid environment. Such a preference might be reflected in the composition of the phospholipid remaining associated with the ATPase after purification. An analysis of the membrane lipids revealed that the three phospholipid classes found associated with both the 38–45 p and gradient purified ATPase preparations (Table 1) corresponded to the three major phospholipid classes found in submitochondrial particles (Krebs *et al.*, 1979). No other phospholipid classes were detected. The relative distribution of the PtdCho, PtdEtn and Ptd₂Gro associated with the ATPase preparations was almost identical with that found in submitochondrial particles even though the phospholipid-to-protein ratios of the 38–45 p ATPase and the gradient purified ATPase were 10% and 5%, respectively, of that observed for submitochondrial particles (Table 1). Assuming the particle weight of the ATPase complex to be 500 000 Da, the number of phospholipid molecules associated

Fig. 1. Diacyl phospholipid stimulation of highly-purified ATPase (a) and oligomycin sensitivity elicited by ATPase reconstitution with various diacyl phospholipids (b)
●, Asolectin; □, $C_{18:1}\text{PtdA}$; ▲, $C_{14:0}\text{PtdGro}$; ○, $C_{18:1}\text{PtdCho}$; ■, $C_{18:1}\text{PtdGro}$

Table 1. *Phospholipid analysis of the mitochondrial ATPase at various ratios of phospholipid to protein*

The phospholipid distribution in submitochondrial particles was calculated from the analysis by Krebs *et al.* (1979). The average values for phosphatidylcholine and phosphatidylethanolamine are from four separate analyses; the values for diphosphatidylglycerol are the average from three separate analyses.

ATPase preparation and phospholipid	Content (nmol phospholipid/ mg of protein)	% of total phospholipid	Fatty acid distribution (mol%)									
			16:0	16:1	18:0	18:1	18:2	20:4	20:5	22:5	22:6	Other
Submitochondrial particles												
Phosphatidylcholine	305	43	26.8	4.1	4.3	17.0	31.9	7.8	1.5	3.0	0.4	3.2
Phosphatidylethanolamine	270	39	2.1	4.4	35.6	4.1	13.2	31.6	4.5	2.2	0.6	1.9
Diphosphatidylglycerol	103	15	2.3	2.7	2.1	6.9	82.0	1.31	0.7	0.8	0.3	1.1
Others	22	3										
Total	700	100										
38–45 p ATPase												
Phosphatidylcholine	29	43	22.6	4.8	9.4	10.6	30.8	6.8	2.3	3.0	3.1	6.3
Phosphatidylethanolamine	28	41	7.0	6.1	34.4	5.9	10.8	25.1	2.5	3.2	2.5	2.3
Diphosphatidylglycerol	11	16	2.4	1.6	5.4	5.7	75.7	0.8	0	2.4	1.6	4.4
Total	68	100										
Gradient purified ATPase												
Phosphatidylcholine	15.6	42	28.1	3.5	7.9	10.8	31.4	6.9	2.1	3.1	3.1	3.3
Phosphatidylethanolamine	16.0	44	4.4	4.5	31.7	6.5	17.1	25.4	3.4	2.3	3.1	1.7
Diphosphatidylglycerol	5.2	14	3.9	1.2	3.0	6.5	77.2	1.4	0	1.8	2.8	2.2
Total	36.8	100										

with the gradient purified ATPase is approx. 8, 8, and 3 for PtdCho, PtdEtn, and Ptd₂Gro, respectively, for a total of 19 per molecule of enzyme.

The fatty acid compositions of the phospholipids associated with mitochondrial inner membrane particles and the two delipidated ATPase preparations are also reported in Table 1. A comparison of the acyl group distribution of the phospholipids remaining with the two delipidated preparations revealed significant differences only in the amount of palmitic acid in PtdCho. The most dramatic difference was a 5.5 mol% increase in the palmitic acid remaining after further treatment of the 38–45p ATPase resulted in an additional removal of 50% of the endogenous phospholipid. The PtdCho C_{16:0} amounts were, however, quite similar in submitochondrial particles and the gradient purified ATPase (26.8 versus 28.1 mol%). Moreover, the fatty acid compositions of phospholipids from the two ATPase preparations were also similar to that obtained for the PtdCho, PtdEtn, and Ptd₂Gro from submitochondrial particles prepared from mitochondrial inner membrane. The most notable differences were 6 mol% decreases in PtdCho C_{18:1} and PtdEtn C_{20:4} in the two ATPase preparations as compared with submitochondrial particles.

Cholate-mediated exchange of mitochondrial phospholipid for exogenous phospholipid in the ATPase

In order to further elucidate the phospholipid requirements of the delipidated ATPase, a deter-

gent-mediated phospholipid exchange technique was employed (see under 'Experimental procedures'). Ideally the gradient-purified ATPase should have been utilized for phospholipid-exchange experiments, since it contains very low levels of endogenous phospholipids. Its usefulness was compromised, however, due to the fact that it contained high levels of residual deoxycholate (193 mol/mol of ATPase) and was not stable under the conditions of the phospholipid exchange experiment. Other workers (Cosson & Guillory, 1979) earlier noted stability problems with the gradient-purified ATPase. For these reasons the exchange procedure was applied to the 38–45p ATPase preparation which contained approximately twice as much endogenous phospholipid. It will be demonstrated that the phospholipid exchange procedure with the 38–45p ATPase resulted in stable ATPase preparations reconstituted with chemically-defined phospholipids which contained endogenous phospholipids at levels as low as those observed for the gradient purified ATPase.

The conditions for the phospholipid exchange procedure were established using asolectin. Since asolectin contains a mixture of phospholipids of varying acyl chain composition exchange could not be demonstrated with this lipid source (see under 'Experimental procedures'). However, asolectin was useful in initial studies because of its ability to induce high levels of oligomycin sensitive ATPase activity (Fig. 1). It therefore served as a

sensitive monitor of the effect of the phospholipid exchange procedure on oligomycin sensitive ATPase activity. For this reason, the resulting asolectin-ATPase complex was characterized with respect to enzyme activity, protein, phospholipid, and detergent content.

Fig. 2 illustrates the banding profile obtained after resolution of the detergent-phospholipid-ATPase mixture on the discontinuous sucrose

density gradient. The ATPase activity located in band 3 was approx. 80% of the total recovered and was 75% oligomycin-sensitive with asolectin as the exchanging phospholipid. The major portion of the phospholipid remained in band 1, which was relatively protein deficient. Band 2 was a diffuse band (Fig. 2a) containing small amounts of phospholipid and protein. Table 2 demonstrates that 80% of the cholate in the detergent-phospho-

Fig. 2. Isolation of the ATPase-phospholipid complex from the detergent-phospholipid-protein mixture by discontinuous sucrose gradient centrifugation

The detergent-mediated phospholipid exchange procedure was performed using asolectin and 5.8 mM-cholate as described in the Experimental procedures section. (a) The banding profile after centrifugation. The dimensions of the centrifuge tube were 1.3 cm $\times$ 6.1 cm. The 25% and 60% designations indicate the concentrations of sucrose in the discontinuous gradient. (b) Analysis of bands for protein, phospholipid, and ATPase activity. The determinations were performed as described in the Experimental procedures section.

Table 2. Removal of sodium cholate by the discontinuous sucrose gradient centrifugation

The phospholipid exchange procedure was performed using asolectin and 5.8 mM-sodium cholate as described in the Experimental procedures section. The cholate determinations were also carried out as described in the Experimental procedures section. The M_r of the ATPase was assumed to be 500000.

	Cholate (nmol/mol of ATPase)	Relative amount of cholate removed (%)
A Before discontinuous sucrose gradient centrifugation		
38-45p ATPase	182	—
38-45p ATPase/asolectin/cholate mixture	495	—
B After discontinuous sucrose gradient centrifugation		
38-45p ATPase	44	76
38-45p ATPase/asolectin/cholate mixture (band 3)	103	79

lipid-ATPase mixture is removed by the centrifugation procedure. The discontinuous gradient was also equally effective in removing residual cholate from the 38–45 p ATPase which has not been supplemented with additional detergent. These results demonstrate the efficacy of the gradient centrifugation procedure in removing the major portion of the cholate utilized to mediate phospholipid exchange.

Fig. 3 is a sodium dodecyl sulphate/polyacrylamide-gel electrophoresis analysis of the phospholipid exchanged ATPase recovered from the sucrose density gradient. Lane 2 shows the intact 38–45 p ATPase which also contains a 30000 Da polypeptide attributed to the adenine nucleotide transport protein (Serrano *et al.*, 1976). Lane 3 is the polypeptide profile of band 3; it demonstrates the known subunits of the F_0 - F_1 ATPase and has relatively high amounts of the 30000 Da polypeptide. This electrophoretic analysis confirms that the phospholipid exchange procedure results in a phospholipid-enzyme complex containing all the known subunits of the oligomycin sensitive ATPase.

To determine quantitatively the replacement of endogenous phospholipid with a defined phospholipid, the exchange procedure was first performed with dioleoylphosphatidic acid ($C_{18:1}$ PtdA) because this phospholipid had elicited relatively high levels of oligomycin-sensitive ATPase activity in reconstitution experiments (Fig. 1). The ATPase phospholipid substitution procedure did result in the replacement of residual mitochondrial phospholipid with $C_{18:1}$ PtdA (Table 3), and the amount of phospholipid substitution was dependent on the sodium cholate concentration. When 5.8 mM-sodium cholate was employed, 12% of the mitochondrial phospholipid found in submitochondrial particles remained associated with the ATPase. This corresponded to substituting 16% of the ATPase endogenous phospholipid with $C_{18:1}$ PtdA. However, when 23.2 mM-sodium cholate was employed, only 6% of the mitochondrial phospholipid found in submitochondrial particles remained associated with the ATPase, which corresponded to exchanging 60% of the ATPase endogenous phospholipid with $C_{18:1}$ PtdA.

The specific activity of the $C_{18:1}$ PtdA-substituted ATPase samples was also related to the sodium cholate concentration employed during the substitution procedure (Table 3). The ATPase specific activity obtained with 5.8 mM-sodium cholate was about 2.5-fold higher than that obtained with 23.2 mM-sodium cholate. The $C_{18:1}$ PtdA-substituted ATPase samples displayed poor oligomycin sensitivity at all sodium cholate concentrations examined (Table 3), which is in contrast with the

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the asolectin-exchanged ATPase

The electrophoresis was performed as described in the Experimental procedures section. Lane 1, standards of known M_r ; lane 2, the 38–45 p ATPase; lane 3, the asolectin-exchanged ATPase recovered from the 25–60% sucrose interface (band 3, Fig. 2a). The gel pattern assignments are based on those determined by Galante *et al.* (1979) and Montecucco *et al.* (1983). The nomenclature is that used by Montecucco *et al.* (1983) with the numbers 1–6 referring to the membrane sector polypeptides of the ATPase. Abbreviation used: DBP, DCCD-binding protein, OSCP, oligomycin-sensitivity-conferring protein.

observation that simple reconstitution of the ATPase with $C_{18:1}$ PtdA (Fig. 1b) resulted in substantially higher levels of oligomycin sensitivity. Low inhibition with DCCD was also observed with the $C_{18:1}$ PtdA-substituted ATPase at each cholate concentration listed in Table 3.

Two pieces of information argue against cholate being responsible for lowered oligomycin activity

Table 3. *Exchange of mitochondrial phospholipid for C_{18:1}PtdA or C_{18:1}PtdCho in the ATPase*

The phospholipid exchange procedure was performed as described in the Experimental procedures section; nmol of phospholipid/mol of ATPase can be estimated by dividing the fatty acid/mg of protein values by 2. Phospholipid in submitochondrial particles = 700 nmol/mg protein. I, ATPase specific activities measured without asolectin in the assays; II, ATPase specific activities measured with 100 µg of asolectin in the assays.

	Fatty acid (nmol/mg of ATPase)		Original SMP phospholipid remaining bound to ATPase (%)	ATPase specific activity		Oligomycin-sensitive ATPase specific activity (%)	
	C _{18:1} PtdA or C _{18:1} PtdCho			I	II	I	II
		Mitochondrial					
Control							
ATPase	—	197	14	2.5	8.2	63	91
Asolectin-ATPase							
11.6mM-cholate	—	—	—	4.6	5.2	62	72
Samples							
C _{18:1} PtdA-ATPase							
5.8mM-cholate	1772	166	12	6.8	6.7	17	20
11.6mM-cholate	1244	106	8	5.4	5.5	15	26
17.6mM-cholate	1421	101	7	4.4	4.7	9	26
23.2mM-cholate	1049	79	6	2.8	2.9	21	24
C _{18:1} PtdCho-ATPase							
5.8mM-cholate	366	176	13	4.3	4.9	64	70
11.6mM-cholate	659	110	8	2.1	5.1	48	66

in the C_{18:1}PtdA exchanged samples. First, identical substitution experiments where asolectin was employed at similar sodium cholate concentrations yielded much higher levels of oligomycin sensitivity (Table 3, control). Furthermore, Table 2 demonstrates that the cholate concentration in the original 38–45p ATPase preparation was actually lowered due to fractionation on the discontinuous sucrose gradient. Since the cholate in the C_{18:1}PtdA-substituted ATPase is likely to be lower than the amounts in preparations with C_{18:1}PtdA added in the simple reconstitution procedure where high oligomycin sensitivity was observed (Fig. 1b), it is unlikely that the lowered oligomycin sensitivity in the C_{18:1}PtdA-substituted ATPase (Table 3) is associated with residual cholate. The subunit composition of the C_{18:1}PtdA-substituted ATPase was examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to determine whether the catalytic subunits (F₁ region) were being dissociated from the membrane subunits (F₀ region) during the exchange process. The Coomassie-Blue stained gel patterns of the C_{18:1}PtdA-substituted ATPase prepared at the four different sodium cholate concentrations (Table 3) were indistinguishable from each other as well as from that of untreated control ATPase (results not shown). Disruption of the F₀–F₁ complex may be occurring, but if so, it is not accompanied by dissociation of F₀ from F₁ during the cholate-mediated exchange with C_{18:1}PtdA.

The phospholipid substitution experiments were also performed with another synthetic phospholipid, dioleoylphosphatidylcholine (C_{18:1}PtdCho), employing two sodium cholate concentrations (Table 3). While a substantial amount of C_{18:1}PtdCho remained associated with the ATPase after completion of the exchange technique, much less was bound to the ATPase as compared with C_{18:1}PtdA (Table 3). The phospholipid exchange procedure did result in the replacement of mitochondrial phospholipid with C_{18:1}PtdCho, with the amount replaced being very similar to that exchanged by C_{18:1}PtdA at equivalent sodium cholate concentrations.

The specific activities of the C_{18:1}PtdCho-substituted ATPase were lower than those of the C_{18:1}PtdA-substituted ATPase when equivalent sodium cholate concentrations were employed, which was to be expected since C_{18:1}PtdA had been shown to be a better activator of the ATPase (Fig. 1a). While the C_{18:1}PtdCho-substituted ATPase exhibited lower specific activity, it possessed a much better oligomycin sensitivity than the C_{18:1}PtdA-substituted ATPase. When almost 50% of the mitochondrial ATPase phospholipid was replaced with C_{18:1}PtdCho, the ATPase specific activity was 50% inhibited by oligomycin. This level of inhibition is comparable with that observed with simple reconstitution with C_{18:1}PtdCho (Fig. 1b).

The specific activity and oligomycin sensitivity of the C_{18:1}PtdCho substituted ATPase could be

Table 4. *Replacement of mitochondrial phospholipid associated with the ATPase during the exchange procedure*
The phospholipid exchange procedure was performed as described in the Experimental procedures section using 11.6mM-sodium cholate. The amount of phospholipid accompanying different preparations of the ATPase complex was slightly variable, accounting for the difference reported in Table 3, as compared with Tables 1 and 4, for untreated control ATPase.

Phospholipid bound to ATPase	Amount of phospholipid (nmol/mg of protein)	Phospholipid bound after C _{18:1} PtdCho exchange (%)
Diphosphatidylglycerol		
Untreated control ATPase	10.6	100
C _{18:1} PtdCho	5.8	55
Phosphatidylethanolamine		
Untreated control ATPase	28.0	100
C _{18:1} PtdCho	14.2	51
Phosphatidylcholine		
Untreated control ATPase	29.0	100
C _{18:1} PtdCho	15.6	53
Total phospholipid		
Untreated control ATPase	67.6	100
C _{18:1} PtdCho	1304	1929

increased appreciably by addition of asolectin (Table 3). The C_{18:1}PtdCho ATPase prepared in the presence of 11.6mM-cholate increased in activity and oligomycin sensitivity 2.5- and 1.4-fold, respectively, when excess asolectin was added to the assay mixtures. This result demonstrates that oligomycin sensitive ATPase activity can be restored in the 38–45p ATPase even after its endogenous phospholipid complement is reduced by half the original amount.

Since almost 50% of the phospholipid remaining with the mitochondrial ATPase could be replaced by C_{18:1}PtdCho while keeping the ATPase active and oligomycin sensitive, a more detailed characterization of the C_{18:1}PtdCho-substituted ATPase was performed. In these experiments, the absolute amounts of the three different phospholipid classes which co-purified with the ATPase (Table 1) were determined after the enzyme had been subjected to C_{18:1}PtdCho exchange. The amounts of mitochondrial PtdCho, PtdEtn, and Ptd₂Gro remaining associated with the ATPase after C_{18:1}PtdCho exchange at 11.6mM-sodium cholate clearly showed that nearly equal percentages of each mitochondrial phospholipid had been replaced (Table 4). These data demonstrate that each of the three mitochondrial phospholipids was exchanged with equal facility by the C_{18:1}PtdCho in the ATPase complex. Assuming the complex obtained from the gradient after the cholate exchange procedure had a particle weight of 500000Da, this particular preparation of the ATPase contained approx. 17mol of endogenous mitochondrial phospholipid. Notably, only 2.9mol of Ptd₂Gro remained with the enzyme complex when 11.6mM-cholate was used in the exchange procedure.

Discussion

Influence of phospholipid headgroup and acyl chain regions on ATPase specific activity and oligomycin sensitivity

The observations in the present study indicate that both the phospholipid headgroup and acyl chain regions exert effects on the highly-purified mitochondrial ATPase. With respect to phospholipid headgroups, higher levels of specific activities were obtained with acidic phospholipids (C_{18:1}PtdGro and C_{18:1}PtdA) compared with the levels observed following reconstitution with a neutral phospholipid (C_{18:1}PtdCho). These findings are in general agreement with those of previous studies where less pure ATPase preparations and phospholipids of mixed acyl chain composition were used (Bulos & Racker, 1968; Dabbeni-Sala *et al.*, 1974; Cunningham & George, 1975). Moreover, the relative order of phospholipid headgroup stimulation (C_{18:1}PtdGro > C_{18:1}PtdA > C_{18:1}PtdCho) noted in this study agrees well with an earlier study (Cunningham & Sinthusek, 1979) in which partially-purified ATPase was reconstituted with PtdGro, PtdA and PtdCho of mixed acyl chain composition. A major difference between these studies is the 10-fold difference in the levels of ATPase specific activity observed, which is due to the higher purity of the ATPase preparation utilized in the present study. Since the PtdGro > PtdA > PtdCho activation capacity with a less pure preparation of the enzyme was shown to correlate strongly with the ionic charge on the activator liposomes (Cunningham & Sinthusek, 1979), it is likely that this particular property

of the phospholipid headgroup region is primarily responsible for the reactivation of the highly-purified ATPase used in this study.

The phospholipid acyl chain region also influenced reactivation of the highly-purified ATPase complex. The findings of this and other studies (Pitotti *et al.*, 1980; Brown & Cunningham, 1982b; Montecucco *et al.*, 1982) suggest that the lipophilic environment provided by the dioleoyl phospholipid derivatives comprise a more favourable environment for reactivation of the highly-purified ATPase compared with their respective dimyristoyl derivatives. This phenomenon occurs regardless of whether the phospholipid headgroup is acidic or neutral. The phospholipid environment surrounding the highly-purified mitochondrial ATPase is not only crucial for stimulating high levels of activity, but also plays a key role in maintaining oligomycin sensitivity. The present study demonstrates that different regions of the phospholipid affect ATPase oligomycin sensitivity. The finding that both the headgroup and hydrocarbon acyl chain regions influence oligomycin sensitivity reflects the importance of both portions of the phospholipid in providing the environment needed for the functional orientation of the ATPase hydrophilic and hydrophobic subunits. Furthermore, there appears to be an interaction between the headgroup and the fatty acid portion in conferring oligomycin sensitivity, since the relative sensitivities observed with C_{14:0}PtdGro and C_{18:1}PtdGro did not correlate with the relative oligomycin inhibition noted previously with C_{14:0}PtdCho and C_{18:1}PtdCho (Brown & Cunningham, 1982b).

In reconstitution studies where the influence of phospholipids on enzyme activity is being measured, the complicating factor of enzyme aggregation in the reconstituted complex should be recognized. It is possible that an enzyme reconstituted with phospholipids, but remaining as an aggregate of enzyme molecules, might have lowered catalytic activity. While the aggregation states of the reconstituted ATPase were not assessed in the present experiments, observations in other studies suggest that the ATPase was not in an aggregated state after being reconstituted with phospholipids. The results of Cherry *et al.* (1978) with bacteriorhodopsin and Bennett *et al.* (1980) with the Ca²⁺-ATPase indicate that enzyme aggregation in reconstituted systems occurs more readily below the transition temperature of the phospholipid. The relatively low activity of the F₀-F₁ in the presence of C_{18:1}PtdCho, reconstituted either by direct addition to liposomes or by detergent mediated phospholipid exchange, is not likely an aggregation phenomenon directed by the physical state of the phospholipid since C_{18:1}PtdCho has a

transition temperature of -22°C (Bennett *et al.*, 1980). Aggregation related to a mismatch in the thickness of the bilayer and the width of the hydrophobic surface in the enzyme (Lewis & Engelman, 1983a) is also unlikely since the phospholipids utilized in this study do not vary greatly in the width of the hydrocarbon portion of their respective bilayer structures (Lewis & Engelman, 1983b). Furthermore, the F₀-F₁ from *Rhodospirillum rubrum* reconstituted into C_{14:0}PtdCho vesicles by taurocholate mediation appeared to exist as a monomer as assessed by neutron small angle scattering measurements on the resulting proteoliposomes (Nawroth *et al.*, 1983). Moreover, beef heart F₀-F₁ reconstituted with PtdCho, PtdEtn, and Ptd₂Gro by using octylglucoside as mediator appeared to be monomeric in the enzyme-phospholipid complexes as judged by electron microscopic analysis of the phospholipid-ATPase complexes (D. Laird & C. Cunningham, unpublished work). The above-mentioned studies do not support the possibility that the activity differences observed as a function of phospholipid type can be explained on the basis of differential aggregation of the enzyme in the reconstituted complexes.

Selectivity of the highly-purified ATPase for mitochondrial phospholipid under phospholipid exchange conditions

As discussed above, the kinetic studies carried out with the highly-purified ATPase (Fig. 1) have verified that the acidic phospholipids will elicit higher activity from the enzyme than do neutral phospholipids. These findings, plus observations made with less pure preparations of the ATPase, suggest but do not prove that the ATPase requires acidic phospholipids for optimal function. In an attempt to determine directly whether the ATPase has preferential affinity for a particular phospholipid, the cholate-mediated phospholipid exchange procedure (Warren *et al.*, 1974) was adapted for use with the mitochondrial ATPase.

Preceding the phospholipid exchange experiments, an analysis of the phospholipids remaining with the ATPase preparation was carried out. These measurements (Table 1) revealed that no minor mitochondrial phospholipids remained with the preparation, demonstrating that the ATPase complex did not have high enough affinity for a minor phospholipid to prevent it from being removed during enzyme purification. The presence of the three major phospholipids with a distribution similar to that of submitochondrial particles, and with similar fatty acid compositions to those of bulk phase phospholipids, also suggests that the ATPase does not selectively bind and retain a particular phospholipid.

The above supposition appears to be supported by the observations made by using the cholate-mediated phospholipid exchange procedure. With this technique we were able to remove approx. 50% of the phospholipid present in the highly-purified ATPase, and it did not matter which phospholipid was being utilized in the exchange procedure. Use of either the negatively charged $C_{18:1}$ PtdA or the neutral $C_{18:1}$ PtdCho resulted in about 50% of the residual phospholipid being removed. The fact that one phospholipid being exchanged was not more effective than the other in removing endogenous phospholipid suggests that the ATPase complex does not associate with one phospholipid type with higher affinity than with another. This conclusion gains support when the phospholipid remaining after the exchange is also considered. The data in Table 4 demonstrated clearly that the three predominant mitochondrial phospholipids were retained equally under conditions which would be expected to facilitate their removal.

These remaining endogenous phospholipids (approx. 17/molecule of ATPase complex; Table 4), appear to be dissociated from the enzyme complex with difficulty, since the exchange procedure was designed such that at equilibrium essentially all the endogenous phospholipid would be exchanged away from the enzyme complex. Furthermore, even more rigorous exchange procedures resulted in no further removal of endogenous phospholipids. Repeating the exchange procedure twice in succession on the same ATPase sample either with $C_{18:1}$ PtdA or $C_{18:1}$ PtdCho did not produce significant additional displacement of residual mitochondrial phospholipid (results not shown). While approx. 17 mitochondrial phospholipids, including three Ptd₂Gro molecules, were retained under our exchange conditions, they are not sufficient to elicit an oligomycin-sensitive activity from the ATPase complex. This is obvious when one considers that the preparation with 2–3 times as much endogenous phospholipid has very little ATPase activity. Moreover, when recombined with defined phospholipid, either by simple reconstitution (Fig. 1) or by the phospholipid exchange procedure (Table 3), the resulting activity is influenced primarily by the phospholipid added; it does not appear to be controlled by the endogenous phospholipid remaining.

The possibility cannot be ruled out, however, that some of the remaining phospholipid molecules are required for expression of activity when the ATPase is reconstituted with exogenously added phospholipids. An example of such a stringent requirement has been demonstrated with cytochrome oxidase. This latter enzyme, delipidated

and then incorporated into detergent micelles of Tween 80, exhibits full electron transport activity only when it contains 2–3 diphosphatidylglycerol molecules/haem aa_3 (Robinson, 1982).

As mentioned earlier in the Discussion, one might expect to see more negatively-charged phospholipid, e.g. Ptd₂Gro, remaining preferentially with the ATPase after the cholate-mediated exchange procedure, since acidic phospholipids elicit higher activity from the lipid-depleted ATPase than do neutrally-charged phospholipids. Two considerations result in a modification of this view, however. First, the highest ATPase activity elicited in experiments *in vitro* may not accurately reflect conditions in the cell where the enzyme may never turn over nearly as rapidly, functioning either as an ATPase or ATP synthetase. Second, if one assumes that the rate *in vitro* of ATPase activity is, indeed, an indicator of the flux through the enzyme *in vivo*, then it is important to recall that in studies where the activating capacity of phospholipids has been assessed, there has never been any evidence suggesting that the enzyme has a higher binding affinity for one phospholipid than another. For example, when the concentration of phospholipid which elicited one-half maximal ATPase activity from the lipid-depleted ATPase complex was compared with the measured V_{max} , no correlation was observed (Cunningham & George, 1975; Cunningham & Sinthusek, 1979); the concentrations required for one-half V_{max} were approximately the same with all phospholipids tested (Cunningham & George, 1975). While the above-mentioned values are admittedly very indirect indicators of the affinity of the ATPase for particular phospholipids, they nevertheless give no indication that the ATPase has a particularly high binding affinity for a superior activator (acidic phospholipid). It would thus appear from the present and earlier studies that a negatively-charged phospholipid can provide the proper environment for a maximally-active mitochondrial ATPase, while being just as dissociable from the surface of the enzyme complex as are the neutrally-charged phospholipids.

This research was supported by NHLBI grant 29136, NIAAA grant 02887 and NC Heart Association grant 1978-79-A-7. C. C. C. is the recipient of NIAAA Research Development Award 00043.

References

- Bennett, J., McGill, K. & Warren, G. (1980) *Curr. Top. Membr. Transport* **14**, 127–164
- Berden, J. & Voorn-Brouwer, M. (1978) *Biochim. Biophys. Acta* **501**, 424–439
- Bligh, E. & Dyer, W. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917

- Brown, R. & Cunningham, C. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, A1827
- Brown, R. & Cunningham, C. (1982a) *Biochim. Biophys. Acta* **684**, 141–145
- Brown, R. & Cunningham, C. (1982b) *Biophys. J.* **37**, 91–93
- Bulos, B. & Racker, E. (1968) *J. Biol. Chem.* **243**, 3901–3905
- Chalvardjian, A. & Rudnicki, E. (1970) *Anal. Biochem.* **36**, 225–226
- Cherry, R., Müller, U., Henderson, R. & Heyn, M. (1978) *J. Mol. Biol.* **121**, 283–298
- Conover, T., Prairie, R. & Racker, E. (1963) *J. Biol. Chem.* **238**, 2831–2837
- Cosson, J. & Guillory, R. (1979) *J. Biol. Chem.* **254**, 2946–2955
- Cunningham, C. & George, D. (1975) *J. Biol. Chem.* **250**, 2036–2044
- Cunningham, C. & Sinthusek, G. (1979) *Biochim. Biophys. Acta* **550**, 150–153
- Cunningham, C., Sinthusek, G., Spach, P. & Leathers, C. (1981) *Alcoholism: Clin. Exp. Res.* **5**, 417–426
- Cunningham, C., Filus, S., Bottenus, R. & Spach, P. (1982) *Biochim. Biophys. Acta* **712**, 225–233
- Dabbeni-Sala, F., Furlan, R., Pitotti, A. & Bruni, A. (1974) *Biochim. Biophys. Acta* **347**, 77–86
- Douglas, M., Finkelstein, D. & Butow, R. (1979) *Methods Enzymol.* **56**, 58–66
- Findlay, E. & Barton, P. (1978) *Biochemistry* **17**, 2400–2405
- Galante, Y., Wong, S. & Hatefi, Y. (1979) *J. Biol. Chem.* **254**, 12372–12378
- Godinot, C., Gautheron, D., Galante, Y. & Hatefi, Y. (1981) *J. Biol. Chem.* **256**, 6776–6782
- Gornall, A., Bardawill, C. & David, M. (1949) *J. Biol. Chem.* **177**, 751–766
- Kagawa, Y. & Racker, E. (1966) *J. Biol. Chem.* **241**, 2467–2474
- Krebs, J., Hauser, H. & Carafoli, E. (1979) *J. Biol. Chem.* **254**, 5308–5316
- Kun, E., Kirsten, E. & Piper, W. (1979) *Methods Enzymol.* **55**, 115–118
- Laemmli, U. (1970) *Nature (London)* **227**, 680–685
- Lewis, B. & Engelman, D. (1983a) *J. Mol. Biol.* **166**, 203–210
- Lewis, B. & Engelman, D. (1983b) *J. Mol. Biol.* **166**, 211–217
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Montecucco, C., Smith, G., Dabbeni-Sala, F., Johannsson, A., Galante, Y. & Bisson, R. (1982) *FEBS Lett.* **144**, 145–148
- Montecucco, C., Dabbeni-Sala, F., Friedl, P. & Galante, Y. (1983) *Eur. J. Biochem.* **132**, 189–194
- Mosbach, E., Kalinsky, H., Halpern, E. & Kendall, F. (1954) *Arch. Biochem. Biophys.* **51**, 402–410
- Nawroth, T., Conrad, H., Vienken, J. & Dose, K. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 923–931
- Papahadjopoulos, D., Jacobson, K., Nir, S. & Isac, T. (1973) *Biochim. Biophys. Acta* **311**, 330–348
- Pitotti, A., Dabbeni-Sala, F. & Bruni, A. (1980) *Biochim. Biophys. Acta* **600**, 79–90
- Robinson, N. (1982) *Biochemistry* **21**, 184–188
- Serrano, R., Kanner, B. & Racker, E. (1976) *J. Biol. Chem.* **251**, 2453–2461
- Warren, G., Toon, P., Birdsall, N., Lee, A. & Metcalfe, J. (1974) *Biochemistry* **13**, 5501–5507